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Neurotoxicity of Asimina Triloba

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NEUROTOXICITY OF *ASIMINA TRILOBA*

By

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ABSTRACT
MYRIAM SANDERS: Neurotoxicity of *Asimina Triloba*
(Under the direction of Dr. Nicole Ashpole)

Neurodegenerative diseases are thought to be brought on by genetic and environmental factors including exposure to compounds and extracts found in plants. Many substances extracted from plants have been shown to induce cell death in neurons. This experiment tests different plant compounds to determine if they induce death in neurons in cell culture. In particular, we focused on extracts and compound from the *Asimina Triloba* plant- a plant that had anecdotally been connected with Parkinson's Disease-like effects. Cultured neurons were treated with increasing concentrations of the compounds and extracts. Twenty-four hours after treatment, viability was assessed. Results show that while most of the compounds and extracts do not induce neurotoxicity, some of the compounds and extracts are neurotoxic and become increasingly more neurotoxic as the concentration of the compounds increase. In the future, it is important to continue exploring the toxicity of this fruit to determine whether consumption is detrimental to neuronal health. Moreover, future studies may elucidate whether these compounds could be used to model degeneration.

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I. Background

Every day, the human body is faced with a daunting range of possible threats that could harm its tissues and cells. Many of these threats are chemical or biological in nature and cause detriment to various body systems. While fascinating and complex, the central nervous system (CNS) is particularly sensitive to damage from chemical and biological toxins and poisons. While there are few exceptions, most of the CNS neurons are unable to regenerate after experiencing toxicity (Xie et al, 2008). Neurotoxicity occurs when neural tissue is damaged or experiences an adverse effect from a chemical agent or biological agent. Subsequently, neurons in the brain or spinal cord can undergo loss of function or structure and eventually die. One critical consequence of neurotoxicity is the onset of neurodegenerative diseases such as Parkinson's Disease or Alzheimer's Disease or other forms of dementia. When neurotoxicity occurs, an individual's symptoms may present as mild onset with memory or coordination problems and over time, these can lead to severe impairments in motor and cognitive abilities which eventually leads to death. Over 6 million people suffer from a neurodegenerative disease. It is estimated that by 2030, this number will double if nothing is done to stop the current trajectory ("The Challenge of Neurodegenerative Diseases", 2018). There are no cures for

neurodegenerative diseases, but some symptomatic treatments have been discovered to slow the development of the diseases (Chen et al, 2012). Neurodegenerative diseases are thought to be brought on by genetic and environmental factors including exposure to compounds found in plants. Environmental stressors can trigger neuronal death and can lead to neurodegenerative disease (Saxena et al, 2011). Many compounds and extracts from plants have been shown to induce cell death in neurons; curare is a well-studied neurotoxin that is derived from the plant, *Chondrodendron tomentosum* (Carl, 2014). The neurotoxic pathway of curare includes subcutaneous injections of the neurotoxin which can result either fatal effects or clinical uses. Extensive research on curare and its neurotoxic properties resulted in discovering clinical uses as a muscle relaxant (Carl, 2014). Additionally, another potential pathway of neurotoxicity is when the plant is ingested into the body and produces negative physiological effects that begin the progression of neurodegenerative diseases. It has been suggested that consumption of plant products may stimulate the onset of neurodegenerative diseases by introducing neurotoxicity to the brain cells.

The plant chosen for analysis of neurotoxicity is *Asimina Triloba*, which is commonly known as the pawpaw tree. Pawpaw trees are among the largest edible fruit trees native to North America. Although they have a tropical appearance, they grow in deciduous forests and temperate climates. The fruit of pawpaw trees are berries and ripen from August to October as they turn from green to brownish-yellow. Many animals and people consume this fruit as it is recently becoming very popular in the United States (Schweitzer, 2017). Production of pawpaw fruit has increased to keep up with demand; these fruits are not sold at grocery stores but can be purchased from farmers' markets or directly from

orchards priced at $15 a pound (Schweitzer, 2017). One county in Maryland hosts a pawpaw festival each year celebrating the fruit. With its newfound fame, the pawpaw tree is well on its way to making it into more and more households in the future. The rise of the pawpaw's popularity has led to increased discussion of the fruit and its properties. There has been research conducted on the antioxidant and phenolic properties of the pawpaw suggesting that it may have the potential to be a natural source of antioxidants (Nam et al, 2017). However, a recent study noted the use of pawpaw twig extracts as anticancer alternative medicine due to decreased cell viability when the extracts were administered to cultured cancer calls (Coothankandaswamy, 2010). This paper discusses potentially toxic compounds within the pawpaw plant. Another, more recent study identified the presence of the compound annonacin in the pawpaw fruit (Potts et al, 2012). Annonacin is an acetogenin molecule known to be toxic to cortical neurons. The study found that the pawpaw does contain a high concentration of annonacin, and that crude fruit extract induces neurotoxicity and that further study is needed. These findings made the pawpaw an attractive candidate for neurotoxicity experimentation in our laboratory.

In recent discussion, a link between the pawpaw and neurodegenerative disease has been suggested; pawpaw consumption may be harmful to humans because the fruit may contain nerve compound toxins. The rise is pawpaw popularity is fueled by discussion on internet and conversations through message boards. Some individuals claim to have experienced adverse neurological effects after consuming the fruit such as headaches and severe sensitivity to light, and warn against eating the skin or leaves of the pawpaw (Kastanie, 2016). Recent scientific papers propose that overconsumption of tea made

from the leaves of these plants could lead to an increased risk of atypical Parkinsonism later in life (Pomper, 2009). Still, others describe intense gastrointestinal distress and allergic reactions post consumption of fruit (Kastanie, 2016). Perhaps cloaked under the seemingly harmless guise of a natural product, the pawpaw fruit could propose a threat to the safety and wellbeing of neurons. Although hypotheses and suggestions have been made about the link between the pawpaw tree and neurodegenerative disease, there has not been significant research conducted on the extracts and compounds of the pawpaw tree, and the question of neurotoxicity remains unanswered. The research conducted in the following experiments sought to examine this idea and to further understand the effects of the compounds and extracts of the pawpaw tree on neurons. If the pawpaw fruit does indeed have neurotoxic properties, then neuron death will occur and be evident in viability assays. We hypothesize that the pawpaw extracts and compounds will display a concentration-dependent decrease in cell viability in cultured neurons.

II. Methods

Neuron prep

The cortical neurons of fetal rats were harvested for cell culture (**Figure 1**). For the plate preparation, 24-well plates were coated in poly-lysine and then washed two times with distilled water and vacuumed. Two petri dishes and five 15 mL conical tubes were placed in ice and cold HBSS was added. Fetuses were removed from the rat and the brains were dissected in the petri dishes. In the dissection process, a cut was made along the midline and horizontally across the occipital bone. Then the brain was separated into hemispheres and the meninges were peeled back to extract the cortex. The cortices were diced and placed in the 15 mL tubes. Papain was added to the tubes to enzymatically digest the tissue for 20-25 minutes at 37 degrees C, and then the papain was washed out two times with media. The cells were then mechanically digested with a series of pipettes. The cells were suspended in a single cell solution of 10 µL of cells per 90 µL of complete neurobasal media with serum to give a 10-fold dilution. The cells were loaded into a hemocytometer and counted to determine the number of cells for plating which is 923 µL of cells per 48 mL of complete neurobasal media with serum. The total number of neurons used was 125,000 per well. 500 µL of the cell solution was plated on 24-well plates and stored in the incubator for twenty-four hours. All 500 µL of the media in each well of the 24-well plates was removed because the media contained serum and then replaced with 500 µL of fresh neurobasal media without serum. The plates were placed back in the incubator until treatment.

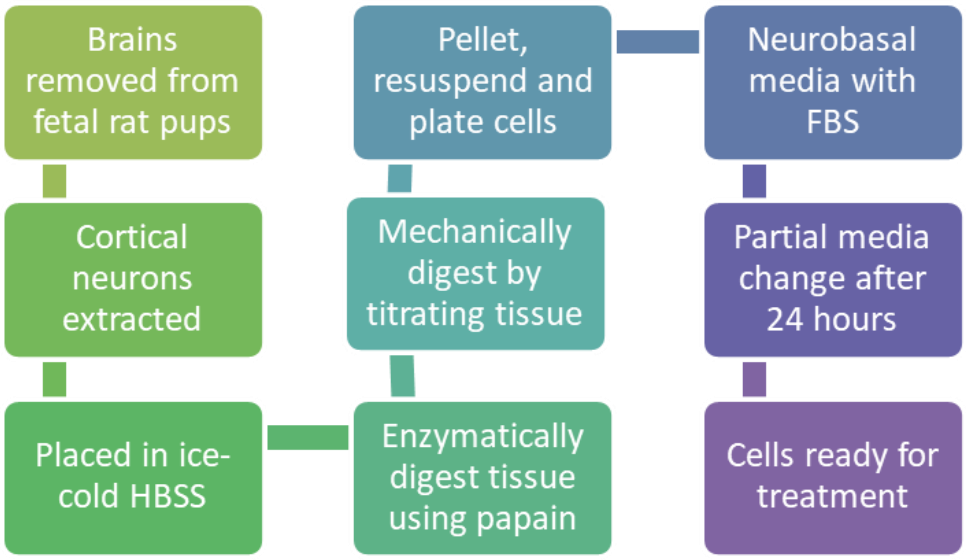

Figure 1: The workflow of cortical neuron culture is shown.

Compound treatment

9 compounds and extracts of the pawpaw were tested for neurotoxicity. They were suspended in a solution of dimethyl sulfoxide (DMSO) at 1000 µg/L concentrations. The compounds and extracts were received from Dr. Iklhas Khan and Taghreed Majrashi in conjunction with the Department of BioMolecular Sciences and the Thad Cochran Research Center for Natural Products. An extract is a combination of many different chemicals from the pawpaw that were released following distinct extraction protocols (methane, heat, ect). A compound is a single, pure chemical isolated from an extract. Due to intellectual property rights, the extracts and compounds are labeled as numbers throughout this report, rather than chemical name/extract identifying information. Four 15mL conical tubes were prepared for each compound and extract with 3 mL of complete neurobasal media. Each of the four conicals received a different concentration of the drug. The drugs were administered in decremental concentrations of 6 µL, 3 µL, 0.6 µL

and 0.006 µL to result in final concentrations of 10 µg/L, 5µg/L, 1µg/L, and 0.1µg/L, respectively. To achieve the lowest concentration (0.1 µg/L), the 1µg/L stock was diluted 1:10 in DMSO. Two controls were included- complete neurobasal media and neurobasal media with DMSO. DMSO was the vehicle for the other extracts and compounds, thus our control had an equal concentration of DMSO as our highest drug treatment. With a total of four conical tubes for each of the nine compounds or extracts and two conical tubes for the controls, there were a total of 38 conical tubes. The contents of the tubes were pipetted into a 24 well plates of 8-10 day old neuron cultures. The plate had been prepared beforehand with complete neurobasal media. To ensure proper mixing of the diluted compounds and extracts in the wells, 250 µL of the media was removed from each well. 250 µL of the diluted drugs in the conical tubes were pipetted into each well. The treated plates were put back into the incubator for 24 hours.

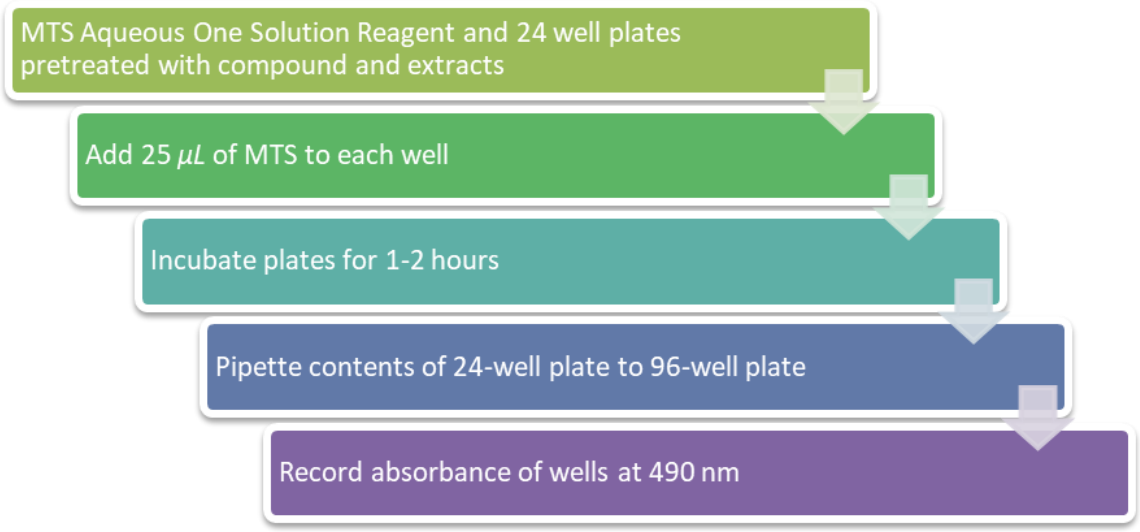

Figure 2: The workflow of MTS assay is depicted.

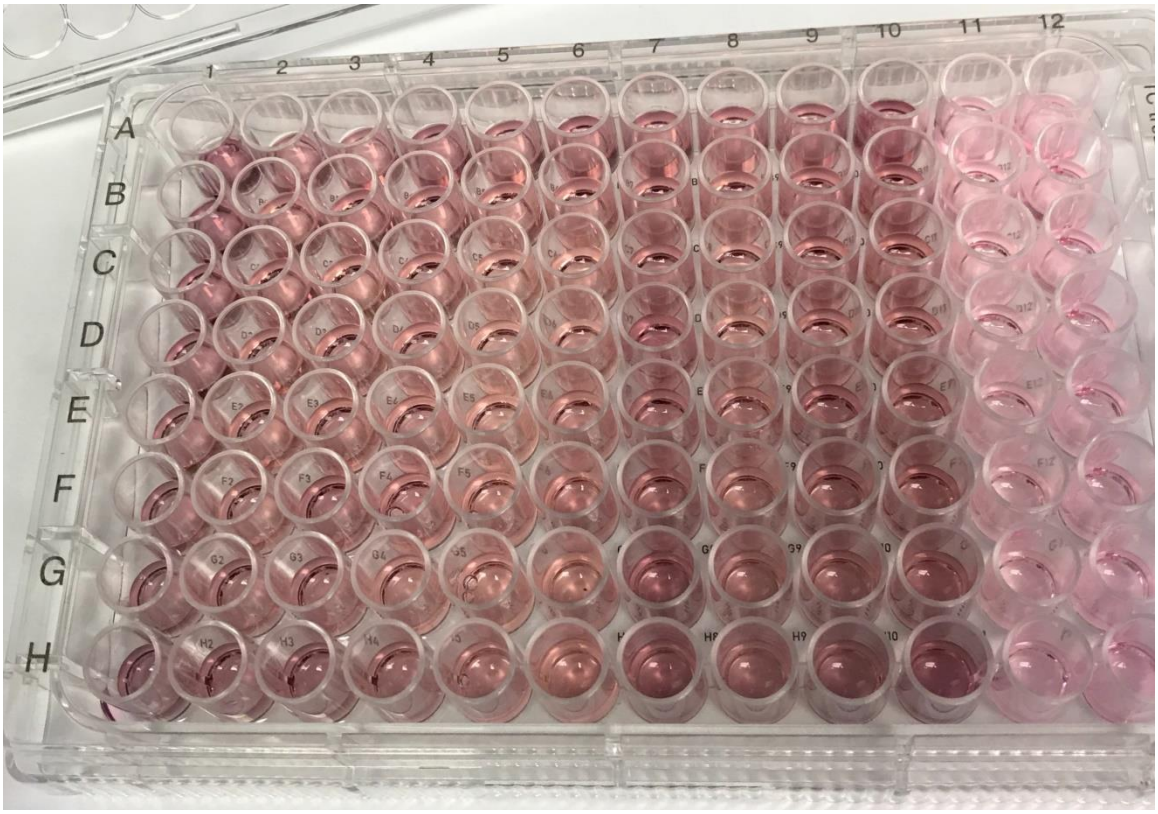

Figure 3: MTS stain of cell culture after two hour incubation.

MTS assay

To assess cell viability, MTS assay was used on the treated plates 24 hours after the drugs were applied (**Figure 2**). The tetrazolium salt used is a measure of metabolized formazan which typically occurs coincident with cell death. MTS assay measures cell viability through assaying the functional metabolism of cells. MTS AQueous One Solution reagent was removed from -20°C freezer and placed in a water bath at 37 °C for ten minutes. 24-well plates with cultured neurons were taken from the incubator, and 25 µL of MTS was added to each of the wells of compounds and extracts on the 24-well plates, per manufacturer's recommendation. Ten wells containing controls were picked at random to receive 25 µL of MTS also. Once the MTS was administered to the wells, the plates were put back into the incubator for 1-2 hours. The color of the solution in the

wells changes from yellow to purple-gray (**Figure 3**). The contents of the wells treated with MTS were moved from the 24 well plate to a 96 well plate. The 96 well plate was put into the plate reader to read the absorbance of the wells at 490 nm.

Cell Culture Immunostaining

The neuronal cell culture was stained using immunofluorescence to ensure the cells were predominantly neurons. To prepare the slide, the coverslips were coated with poly-L-lysine for an hour at room temperature. Afterwards, the coverslips were rinsed with sterile water three times and then dried completely, sterilized, and rinsed briefly in phosphate-buffered saline. Cells were then cultured on the coverslips, as described above.

At 8-10 days of culture, the cells were fixed with 4% paraformaldehyde in phosphate buffer pH 7.4 for ten minutes at room temperature. The cells were washed three times with ice-cold PBS. For blocking and immunostaining, the cells were incubated with 1% BSA, 22.52 mg/mL glycine in PBS + 0.1% Tween 20 (PBST) for thirty minutes to block unspecific binding of the antibodies. The cells were incubated in diluted antibody in 1% BSA in PBST for an hour at room temperature. The solution was decanted and the cells were washed three times in PBS for five minutes each time.

The primary antibody used was beta tubulin rabbit monoclonal antibody. The cells were incubated with the secondary antibody which was Cy5 anti-rabbit antibody. Cy5 is a fluorophore and has a far red color. The secondary antibody solution was decanted, and the cells were washed three times with PBS for five minutes each in a dark environment. 100 µL of the cell and antibody solution was pipetted onto parafilm. The 100 µL dots were inverted onto a coverslip. Once the cells were mounted, the coverslips

were stored in a dark room a 20°C. A Nikon Ti2 inverted microscope was used to view and photograph the neurons (**Figure 4-6**).

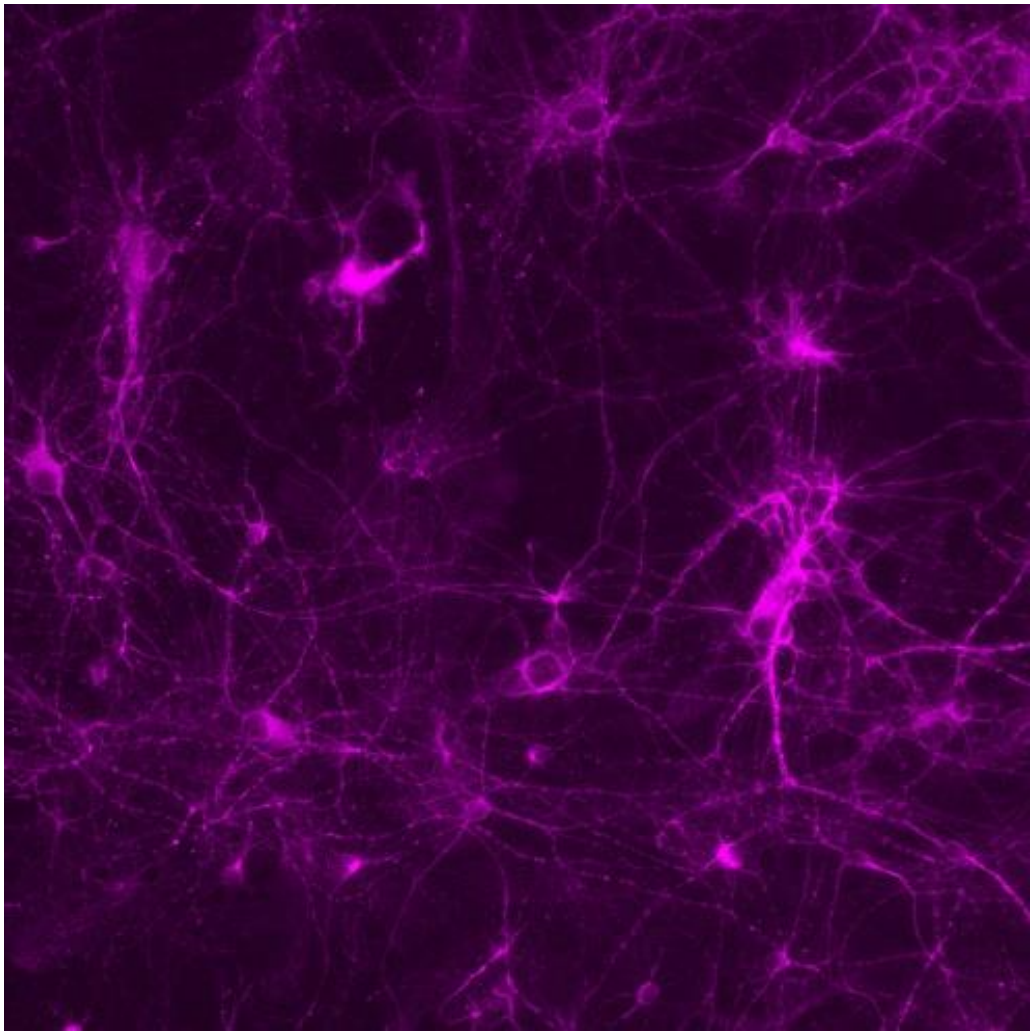

Figure 4: Neuronal Cultures: Primary cultures of neurons were immunostained for imaging. The cells were stained with primary antibodies against beta-tubulin, and secondary antibodies with Cy5 fluorescence.

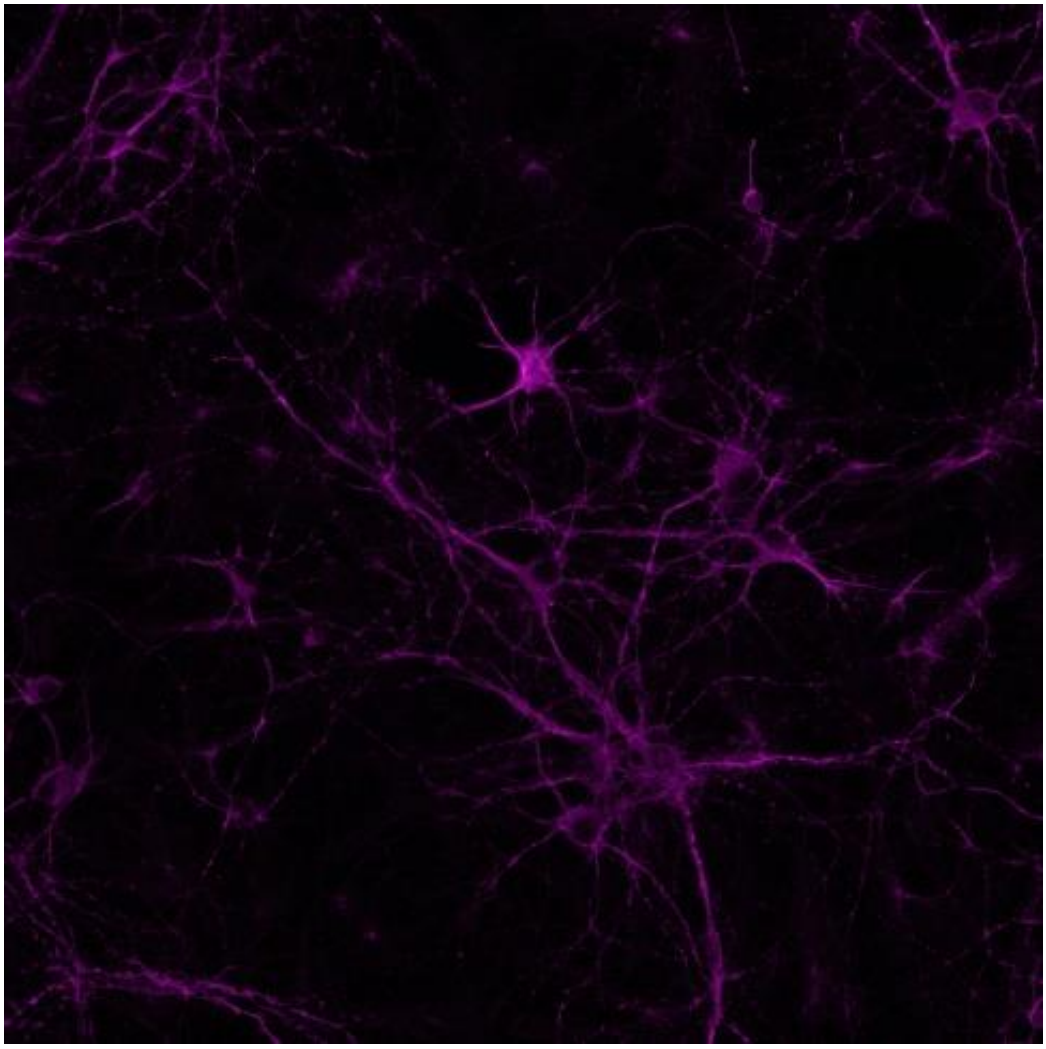

Figure 5: Neuronal Cultures: Primary cultures of neurons were immunostained for imaging. The cells were stained with primary antibodies against beta-tubulin, and secondary antibodies with Cy5 fluorescence.

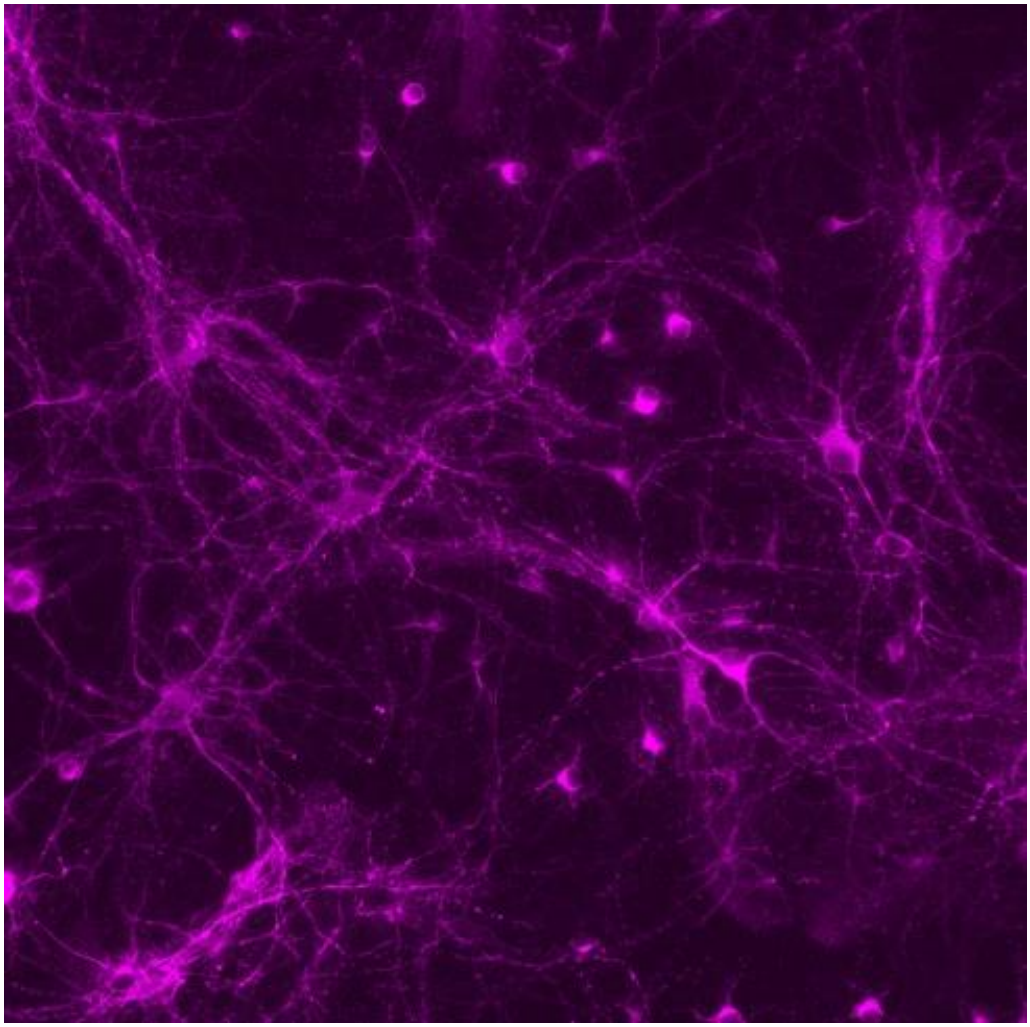

Figure 6: Neuronal Cultures: Primary cultures of neurons were immunostained for imaging. The cells were stained with primary antibodies against beta-tubulin, and secondary antibodies with Cy5 fluorescence.

Data Analysis

The data was collected from the plate reader and transferred to Excel. Each treatment was added to 8 wells, and the entire assay was repeated 3 independent times. Viability was normalized to control for each of the independent assays. Sigmaplot was used to analyze the variance and experimental differences as well as to graph the data. The data was graphed with Sigmaplot and normalized to the control. Data was shown as mean ± SEM. A one-way ANOVA was used to perform statistical analysis before the Dunnett's post hoc test to examine the significant different against the control at $p<.05$ for each dose of a compound or extract. The error bars are plus or minus one standard error measurement, and the data is plotted in linear form.

III. Results

We established neuron cultures from rat cortices and grew cells in vitro for 8-10 days prior to treatment. We verified that they were neurons using MAP2 and beta tubulin staining (**Figures 4-6**). After development in culture, neurons were treated with extracts and compounds isolated from *Asmina triloba*, the pawpaw tree. These extracts and compounds were provided by the National Center for Natural Product Research.

Twenty-four hours following treatment, we analyzed cell viability. Results show that some of the compounds and extracts are neurotoxic and become increasingly more neurotoxic as the concentrations increase. Each graph represents 6-8 wells of neurons tested in 3 independent biological replicates. In particular, Extract 1 and 2, and Compound 1, 2, 4, and 5 showed statistically significant neurotoxic effects on neurons based on data from MTS assay. Extract 1 displays a significant decrease in cell viability at 1 µg/L. Cell viability levels off at 5 and 10 µg/L **(Figure 7)**. Similarly, Extract 2 also shows a significant cell viability decrease at 1 µg/L and has the same leveling off pattern **(Figure 8)**. Extract 3 does not have a significant decrease in cell viability according to statistical analysis **(Figure 9)**. The graph for Extract 3 shows slight decrease in cell viability after 1 µg/L and then consistent cell viability at 5 and 10 µg/L. Compound 1 displays a significant dose-dependent decrease in cell viability **(Figure 10)**. A concentration of 0.1 µg/L could not be tested because we ran out of this compound. A

dose concentration of 1 µg/L shows an immediate drop in cell viability when compared to the control. Increasing concentrations of doses do not drop cell viability further. On the graph, a plateau is displayed. For compound 2, the decrease in cell viability is significant but not as plunging as Compound 1. Compound 2 experiences loss of cell viability at 0.1 µg/L and 1 µg/L, and increasing concentration doses of 5 and 10 µg/L only reduce cell viability slightly **(Figure 11).** For Compound 3, there was not a significant decrease in cell viability after statistical analysis with ANOVA **(Figure 12).** The graph shows that increasing dose concentration does not cause a decrease in cell viability and instead a slight increase in cell viability can be seen at 5 and 10 µg/L concentration. While we cannot explain this variation, it is repeated over all three replicates. Compound 4 displays a significant decrease in cell viability at a concentration of 10 µg/L **(Figure 13)**. Cell viability remains constant at 1 µg/L but sharply decreases after 5 µg/L. Compound 5 also has significantly decreased cell viability at 1 µg/L and remains decreased at 5 and 10 µg/L **(Figure 14).** For Compound 6, there was no significant decline in cell viability according to statistical analysis with ANOVA **(Figure 15)**. After 5 µg/L, cell viability decreases slightly. Again, this variation cannot be explained, but it was repeated over all three biological replicates. To easily summarize the data, all of the extracts and compounds were combined in a bar graph to show 10 µg/L concentrations normalized to the control **(Figure 16)**. This graph shows variation of each compound and extract from the control. Asterisks designate statistically significant decreased cell viability for Compound 1, 2, 4, 5, and Extract 1 and 2. These results were confirmed by Dr. Ashpole in a different cell viability assay: a live-dead assay.

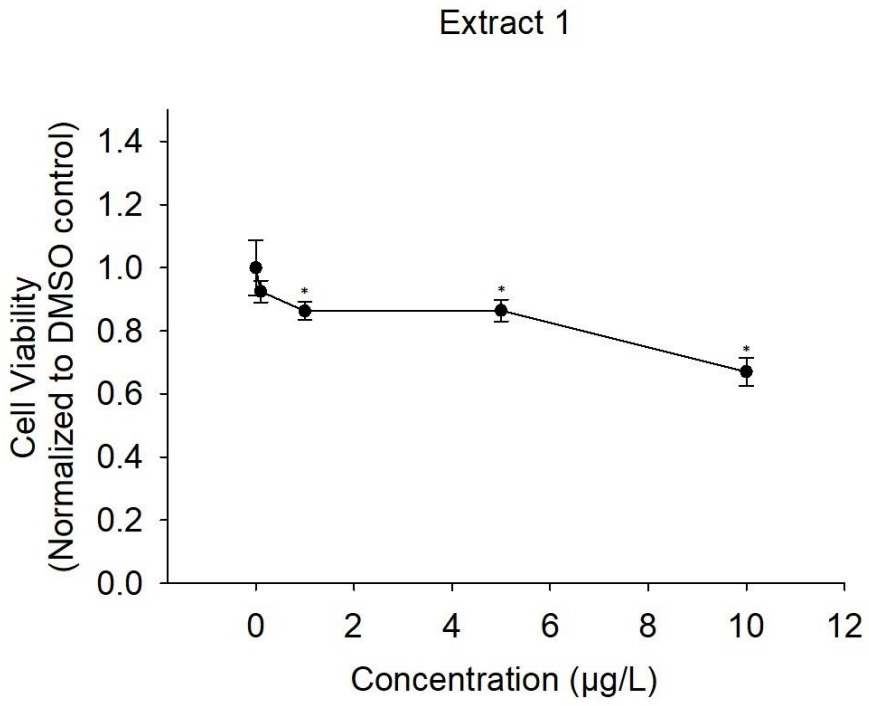

Figure 7: Extract 1 shows a significant concentration dependent decrease in cell viability.

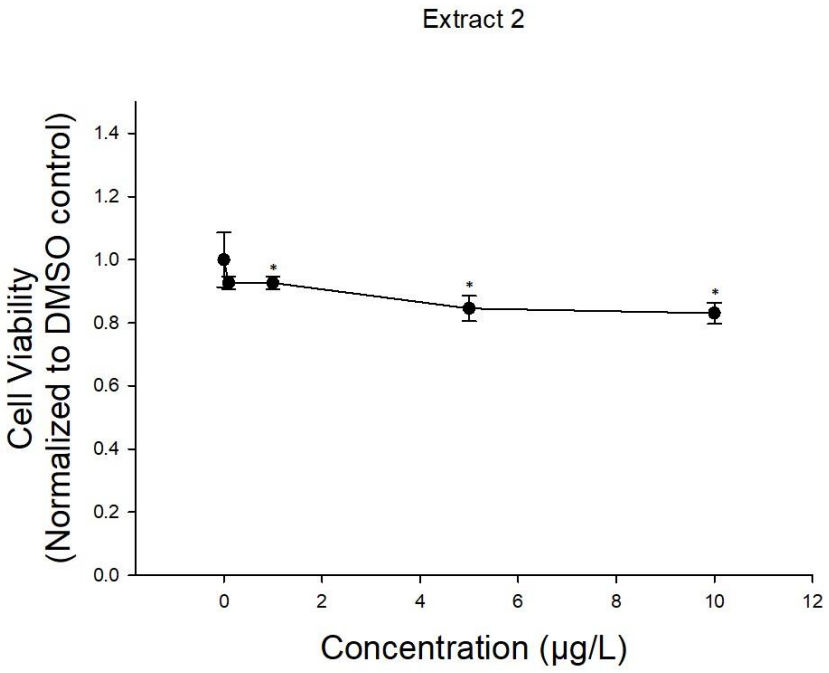

Figure 8: Extract 2 shows a significant concentration dependent decrease in cell viability.

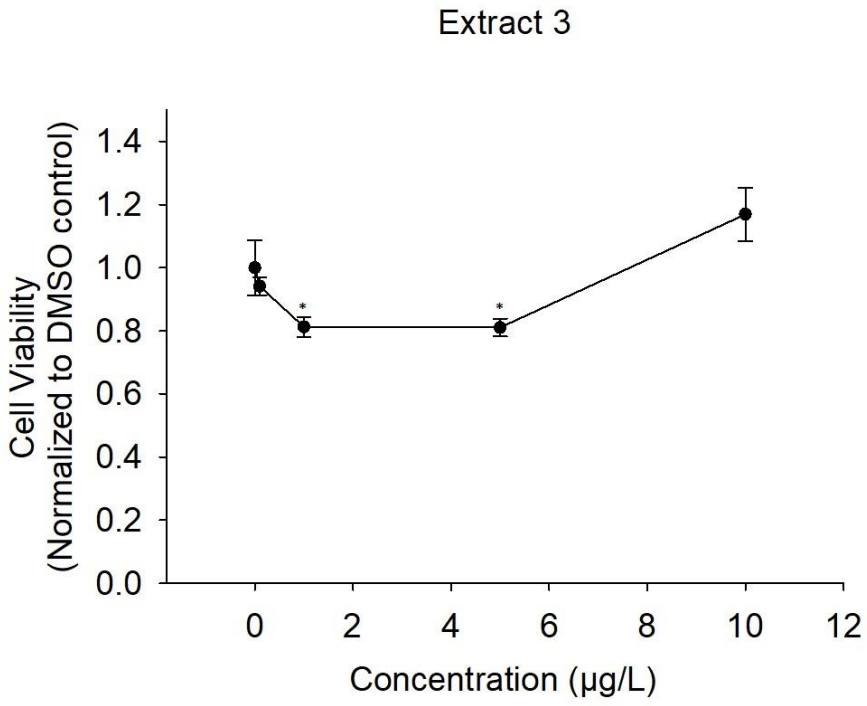

Figure 9: Extract 3 does not show a significant concentration dependent decrease in cell viability.

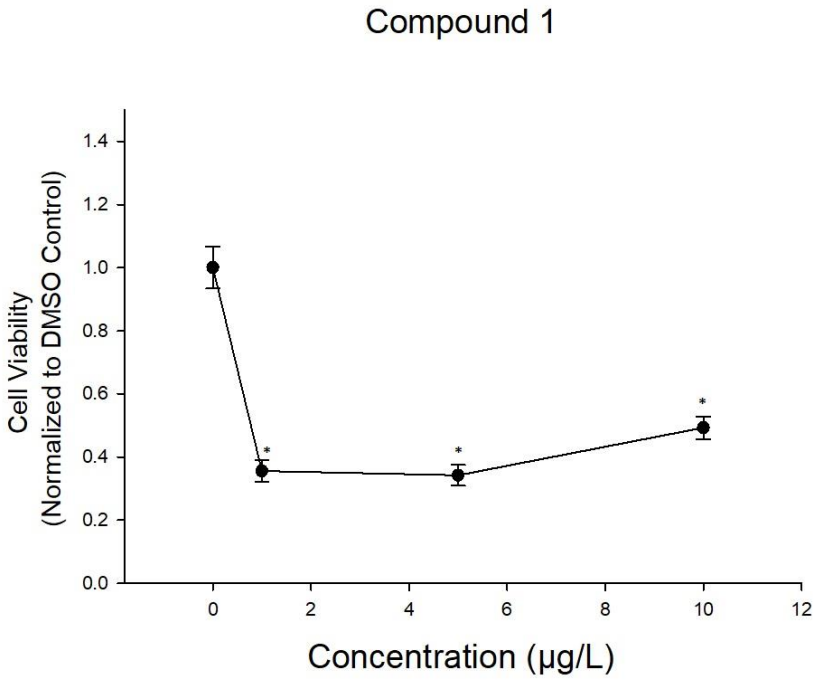

Figure 10: Compound 1 shows a significant concentration dependent decrease in cell viability. A concentration of 0.1 µg/L could not be tested because we ran out of this compound.

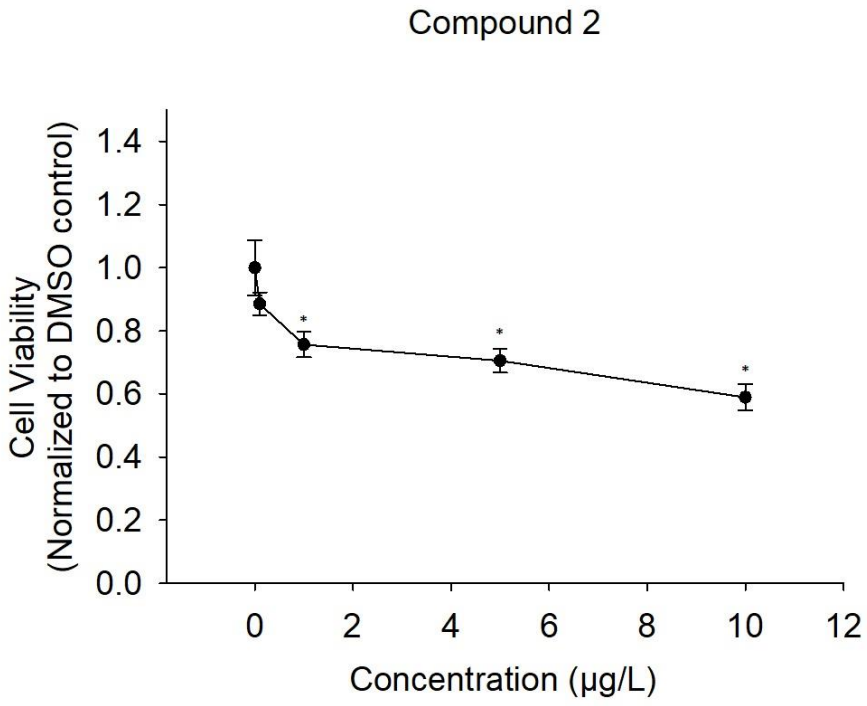

Figure 11: Compound 2 shows a significant concentration dependent decrease in cell viability.

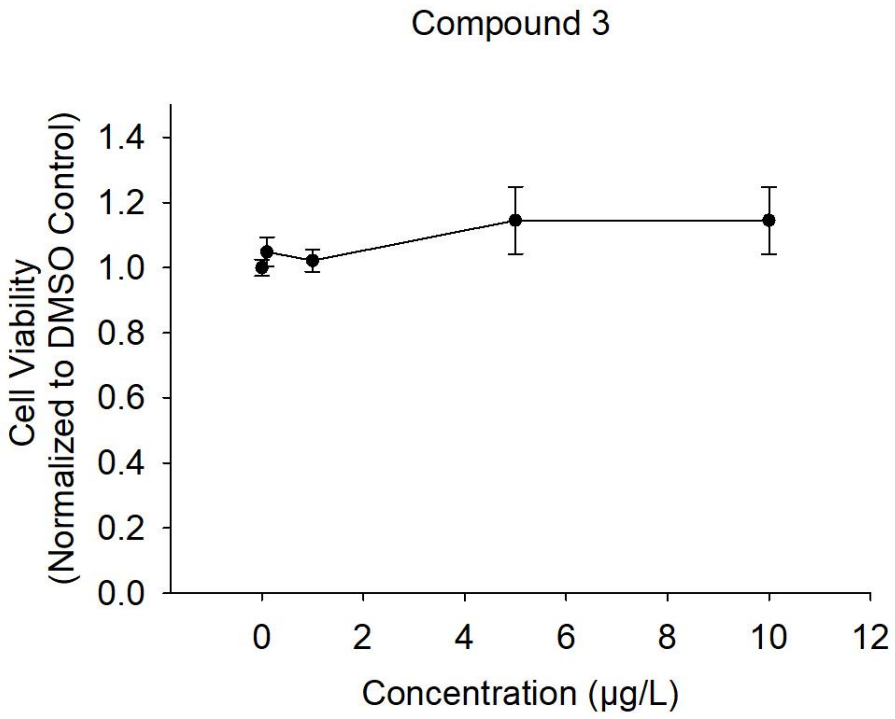

Figure 12: Compound 3 does not show a significant concentration dependent decrease in cell viability.

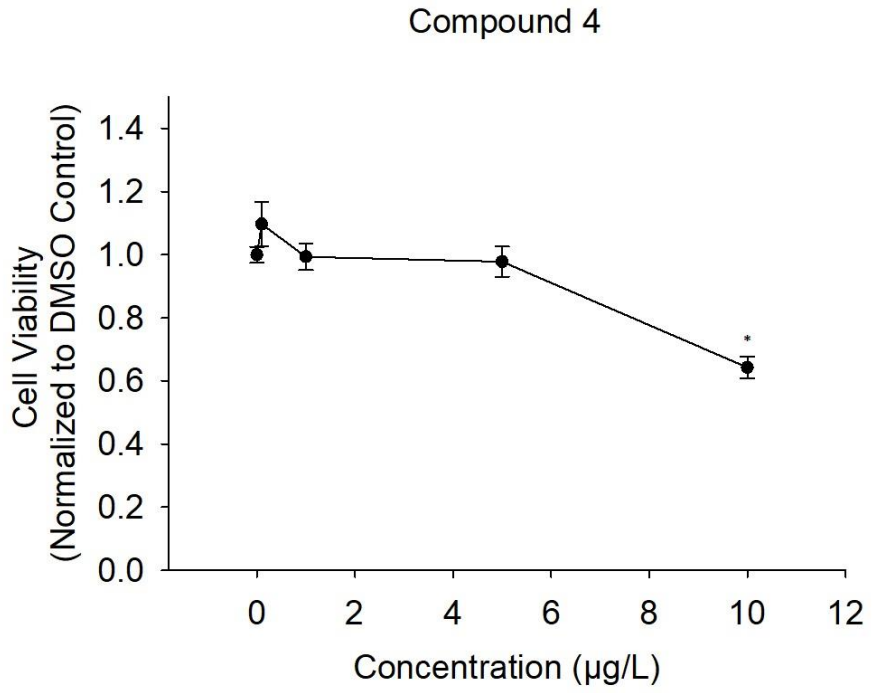

Figure 13: Compound 4 shows a significant concentration dependent decrease in cell viability.

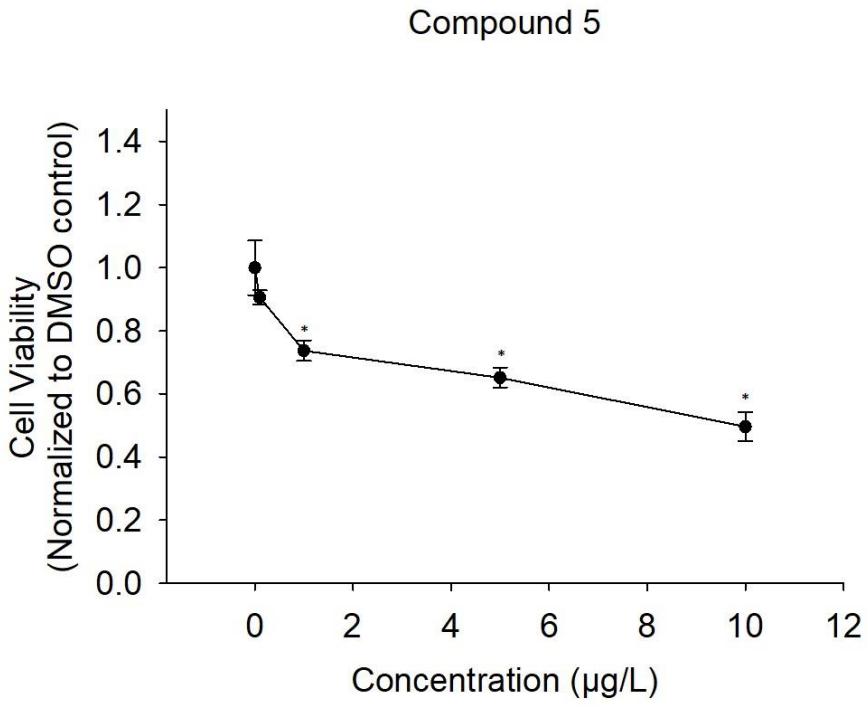

Figure 14: Compound 5 shows a significant concentration dependent decrease in cell viability.

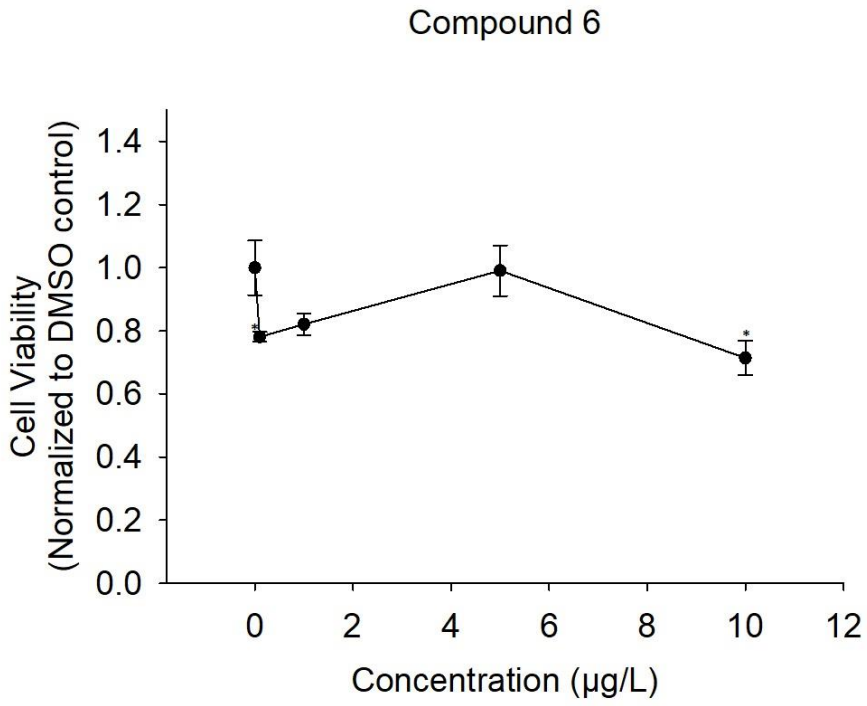

Figure 15: Compound 6 does not show a significant concentration dependent decrease in cell viability.

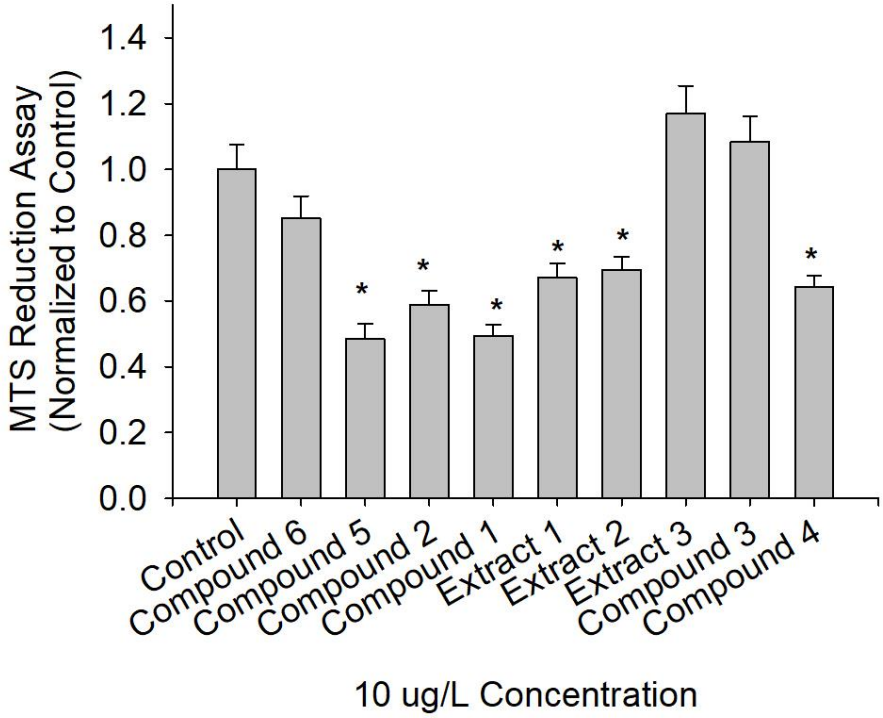

Figure 16: 10 µM concentration of all of the compounds and extracts were normalized to the control. Asterisks designate statistically significant decreases in cell viability.

IV. Discussion

Conclusions

Consistent with our hypothesis, compounds and extracts from the pawpaw tree have the capability to induce neuronal death. Some compounds and extracts displayed a significant decrease in cell viability as concentration increases, yet others showed no difference or abnormal dose-response curves. Investigating the neurotoxicity of the pawpaw is important because of significant possibilities: creating a non-neurotoxic pawpaw and using potential pawpaw neurotoxins for cancer research. The wonderful advances of science and technology have allowed for modification of plants which can result in altered fruit. If annonacin is the sole reason for the neurotoxicity of the pawpaw, varieties with less annonacin can be breed to create a less harmful fruit. Annonacin has been studied as a potentially neurotoxic agent in many papers. Research has been done to develop a method for quantifying this environmental neurotoxin in rat brain homogenates (Bonneau, 2016). Annonacin is the most abundant acetogenin found in the pawpaw, and it was administered orally and intravenously to rats to quantify the amount that crossed the blood brain barrier. This study found that a very weak proportion of the administered amount was detected in the rats' brains; about 3% of annonacin administered was shown to be bioavailable. This brings up the possibility of further research on other toxins in the pawpaw to be quantified in rat brains. Another paper examined the other acetogenins in the pawpaw; over 40 have been identified (He, 1997). The cytotoxic properties of newly discovered acetogenins can be modulated for cancer research as antitumor agents. One study found that the pawpaw contains an average annonacin concentration of 30 µg/L (Levine, 2015). Their research also analyzed the pawpaw for squamocin, another

acetogenin which could contribute to the potential neurotoxicity of the pawpaw. The genes responsible for chemical synthesis within plants like the pawpaw have been characterized in other studies. Perhaps modification of the pawpaw by altering the gene responsible for acetogeninin synthesis may allow for breeding of new varieties with fewer acetogenins or reduced concentrations of these toxins in the fruit.

Additionally, clinical uses of the pawpaw may be identified by modulating the neurotoxic pawpaw compounds and extracts such as annonacin for cancer research (McLaughlin, 2008). One study isolated three acetogenins from the stem bark of the pawpaw to assess for bioactivity against six human solid tumor cell lines to possibly identify antitumor agents (He, 1997). Compounds 1 and 2 in this experiment were found to show potent activities against the tumor cell lines. This study noted that the mechanism of selective cytotoxicity is through inhibiting ATP production through inhibition of mitochondrial complex 1. Another study isolated two acetogenins: asitrilobins C and D from the pawpaw seed to test for cytotoxicity on breast carcinoma and colon adenocarcinoma, and found the mechanism of cytotoxicity to be through both the inhibition of mitochondrial electron transport (complex I) and the inhibition of the plasma membrane NADH oxidase (Woo, 2000). The effects of these compounds on neuronal-based tumor lines have not been published, but our evidence suggests that perhaps compounds from the pawpaw would be beneficial for killing these cancer cells as well.

The research conducted in these experiments analyzed the effects of the pawpaw tree extracts and compounds on neurons and sought to better understand how neurodegenerative diseases are brought on by environmental factors. Crude fruit extracts and isolated compounds can induce neurotoxicity and further experimentation is needed

to determine these effects. The research conducted in this experiment is not conclusive on the neurotoxicity of the pawpaw.

Limitations of Experiment and Interpretations

This study is not without limitations particularly in regards to the high concentration that resulted in neurotoxicity in cultured neurons. The MTS assay used to determine cell viability is not a direct measure of cell death, although the functional metabolism measured occurs coincident with cell death. 10 µg/L is a high concentration that is unlikely to be achieved in average consumption of pawpaw fruit. Furthermore, it is not known if the toxins actually cross the blood-brain barrier; additional research is needed to determine this.

Future Studies

Further studies can be conducted to explore the toxicity of the pawpaw to determine if consumption is detrimental to neuronal health. Extensive research could be done to assess the level of toxins in the pawpaw like annonacin to provide more information regarding the neurotoxicity of the individual pawpaw compounds and extracts. Also, further research can be done with a live-dead assay at the concentrations that were most efficacious using MTS because annonacin may inhibit mitochondrial complex 1 which would reduce MTS metabolism, potentially independent of cell death (Bonneau, 2016; Schapira, 2010). Additionally, possible research could include administering these neurotoxic compounds and extracts to animals to model disease degeneracy because neurodegenerative diseases affect neurons at varying rates of progression. Modeling neurotoxic progression with an animal study could provide more information for therapeutic advances to combat the debilitating effects of neuronal death.

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